ONLINE SUPPLEMENT

# CYP4A2-Induced Hypertension is 20-HETE and Angiotensin II-Dependent

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*Running Title*: Ang II mediates 20-HETE-induced hypertension

Key words: Cytochrome P450; 20-HETE; ACE, Angiotensin II, Hypertension

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METHODS AND MATERIALS

***Materials.*** 20-HETE (20-hydroxy-5,8,11,14-eicosatetraenoic acid), 20-HEDE (20-hydroxyeicosa-6(Z),15(Z)-dienoic acid) and HET0016 (*N*-hydroxy-*N*α-(4-butyl-2-methylphenyl)-formamidine) were prepared by chemical synthesis as previously described [1-3].

***Cell culture***. Human umbilical vein endothelial cell-derived Ea.hy926 cells [4] were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing hypoxanthine-aminopterin-thymidine supplement (Sigma-Aldrich), 5% Penicillin/Streptomycin (Invitrogen) and 10% Fetal Bovine Serum (FBS, USA Scientific). Human microvascular endothelial cells (HMVECs) were obtained from Invitrogen and grown in Medium 131 containing microvascular growth supplement (Invitrogen) and 10% FBS. Passages 3-5 were used. All cells were maintained at 37ºC in a humidified incubator with an atmosphere of 5% $CO_2$. Cells were cultured on 6-well plates and starved in serum-free media for 24 hours at 80-90% confluency. On the day of the experiment, cells were incubated in the presence and absence of 20-HETE (1 or 5 nmol/L) and 20-hydroxyeicosa-6(Z),15(Z)-dienoic acid (20-HEDE, 5 nmol/L) for 30 min (for gene microarray) or 2 hours. After three washes with 1X PBS, cells were trypsinized, centrifuged, and lysed in 350 µl Buffer RLT (Qiagen) for RNA isolation. A gene microarray (GEarray microarray, SA Biosciences) was performed on mRNA extracted from HMVECs. Real-time PCR was performed on mRNA extracted from Ea.hy926 cells.

***Animal Experimentation***. All experimental protocols were performed following an IACUC-approved protocol in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*. Sprague-Dawley rats (9 to 10 weeks old, 225-250 g body weight) were used in all experiments. Rats were fed normal chow diet and had access to water ad libitum. Vascular endothelium was targeted by lentiviruses carrying the CYP4A2 or GFP constructs under the control of the endothelium specific promoter VE-cadherin (VECAD-4A2 and VECAD-GFP, respectively) (Lentigen, Baltimore, MA) as previously described [5]. Lentiviruses were injected into the left renal artery (200 µL of $10^9$ TU/mL in saline) and the tail vein (75 µl of $10^9$ TU/mL in saline) under anesthesia with sodium pentobarbital (65 mg/kg body weight, ip). The following experimental protocols were used: *Protocol 1:* The experiment was carried out for 45 days. Rats (n=16) were divided into four groups: A) rats were injected with lenti-VECAD-CYP4A2 at day 0; at day 27, losartan (100 mg/kg/day) was given in the drinking water for 3 days. B) rats were injected with lenti-VECAD-CYP4A2 at day 0 and co-treated with daily injections of the CYP4A selective inhibitor HET0016 (10 mg/kg body weight/day) for the duration of the experiment. C) rats were injected with lenti-VECAD-GFP at day 0. D) rats were injected with lenti-VECAD-GFP at day 0 and co-treated with daily injections of HET0016. *Protocol 2:* The experiment was carried out for 37 days. Rats (n=16) were divided into four groups: A) rats were injected with lenti-VECAD-GFP at day 0; B) rats were given lisinopril (10 mg/kg/day) in the drinking water 3 days prior to

a bolus injection of lenti-VECAD-GFP and thereafter; C) rats were injected with lenti-VECAD-CYP4A2 at day 0; at day 10, lisinopril (10 mg/kg/day) was given in the drinking water for 9 days (day 19 of the experiment); D) rats were given lisinopril (10 mg/kg/day) in the drinking water 3 days prior to a bolus injection of lenti-VECAD-CYP4A2 and 10 days after lentivirus injection; at day 24 of the experiment, the 20-HETE antagonist, 20-HEDE (10 mg/kg/day), was administered intraperitoneally till the end of the experiment. Systolic blood pressure was monitored by the tail cuff method before lentiviral injection and during the length of the experiment. Values within ± 10% of their mean blood pressure measurements were obtained. At the end of all experiments, rats were euthanized and kidneys, aortae and renal interlobar arteries were collected for biochemical analyses.

***Measurement of Plasma/Tissue Angiotensin II, PRC and ACE.*** For measurements of ACE activity, 250 µl of venous blood sample were collected at room temperature without the addition of anticoagulants. Blood was centrifuged at 2500 g for 10 min at 4°C to separate the plasma, and stored at -80°C. For measurements of plasma renin concentration (PRC), 300 µl of venous blood were collected into ice-cold EDTA tubes. Blood was centrifuged at 2500 g for 10 min at 4°C. The plasma was transferred into a pre-chilled conical centrifuge tube and centrifuged again for 10 min at 4°C, and stored at -80°C. For measuring angiotensin II, 0.1 ml of 0.1 µg/ml rat renin inhibitor (AnaSpec Inc.) in ultrapure water (New England Reagent Laboratory) and 0.15 ml of angiotensin inhibitor were added to a pre-chilled EDTA-treated tube. Venous blood (3 ml) was added to the tube and the mixture was gently mixed. Samples were centrifuged at 2000 rpm for 10 min at 4°C. The plasma was transferred into a pre-chilled conical centrifuge tube, centrifuged again for 10 min at 2000 rpm at 4°C, and stored at -80°C. All measurements were carried out by the Hypertension Core Lab at the Hypertension and Vascular Research Center, Wake Forest University.

***Real time PCR***. Total RNA was isolated from aorta and cell samples using the RNeasy Mini Kit (Qiagen) and quantified with NanoDrop (ThermoScientific). RT reaction of total RNA (500 ng) was performed using the Superscript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. Specific primers were designed based on published sequences (Gene bank). PCR primer sequences were as follows: angiotensin converting enzyme (ACE, rat): sense 5'- CGG GAC GTG GCC ATT ATA TT- 3', antisense 5'- CTG CCT CCC AAC GAG TTA GA -3'; ACE (human): sense 5'- CGC TGA AAC CGC TGT ACG A -3', antisense 5'- TGG GGG AGT TGT ACC AGG AG -3'; angiotensin type 1 receptor ($AT_1R$): sense 5'- TAT CAC AGT GTG CGC GTT TC -3', antisense 5'- TGG TAA GGC CCA GCC CTA T -3'; angiotensin type 2 receptor ($AT_2R$): sense 5'- CCT TCC CTG TAT TGT TTC GTT -3', antisense 5'- CGG CAA GAC ATA GTC TCT CT -3'; 18S rRNA (rat): sense 5'- GAT GGG CGG CGG AAA ATA G -3', antisense 5'- GCG TGG ATT CTG CAT AAT GGT -3'; 18S rRNA (human): sense 5'- GAT GGG CGG CGG AAA ATA G -3', antisense 5'- GCG TGG ATT CTG CAT AAT GG -3'. Quantitative Real-Time PCR was performed using the Quantitect SYBR Green PCR Kit (Qiagen) and the Mx3000p Real-Time PCR System (Stratagene).

***Western Blot analysis.*** Frozen interlobar arteries and aortae were pulverized under liquid nitrogen and homogenized in 10 mmol/L potassium phosphate buffer, pH 7.2, containing 25 mmol/L sucrose, 0.1% NP-40, 1 mmol/L EDTA and a cocktail of proteinase inhibitors. The homogenates were centrifuged at 1000g for 15 min and the supernatant was used for Western blot analysis. Proteins (20-30 µg) were separated by SDS/polyacrylamide gel electrophoresis at 75 V for 2-3 h at room temperature and then transferred electrophoretically to a nitrocellulose membrane. Membranes were incubated with the following primary and secondary antibodies: mouse anti-ACE1 monoclonal antibody (1:500; Abcam, ab11734), rabbit anti-AT1 polyclonal antibody (1:500; Abcam, ab18799), rabbit anti-AT2 polyclonal antibody (1:800; Abcam, ab19134), goat anti-rat CYP4A1 polyclonal antibody (1:3000; Daiichi Chemical Co, Japan), goat anti-rabbit IgG (1:2000) and goat anti-mouse IgG (1:1000). Immunoreactive proteins were detected using the ECL Plus detection system (Amersham Life Sciences) according to the manufacturer's instructions. Anti β-actin was used to normalize for loading variations.

***LC-MS/MS analysis of vascular and plasma 20-HETE, EETs, and DHETs.*** Renal interlobar arteries were incubated in 1 ml oxygenated Krebs buffer (37°C, 60 min) with 1 mmol/L NADPH. Reactions were terminated by acidification to pH 4 with acetic acid. Thereafter, 500 pg of internal standard mix (d11-11,12-DiHETrE; d11-8(9)-EET; d6-20-HETE, d8-11(12)-EET )were added as internal standards to each sample. 20-HETE was extracted with twice the volume of ethyl acetate (3 times), dried under nitrogen and stored at -80°C for LC/MS/MS analysis. NaOH (1 mol/L, 100 µl) was added to the arteries after extraction for their dissolution and determination of protein concentration (BioRad, Hercules, CA). For measurements in plasma, acidified water (pH 4) and 1 ng of internal standard mix were added to thawed rat plasma (100-300 µl) and first extracted with 5 vol chloroform/methanol (2:1) followed by addition of 2x1 vol chloroform. The chloroform layers were combined dried under nitrogen. Alkali hydrolysis was performed by adding 1 ml 1M NAOH to each sample followed by 90 min incubation at room temperature. Samples were neutralized with 1M HCL and loaded onto preconditioned Strata-X columns (Phenomenex, Torrance, CA), washed with 2 ml 10% methanol, and eluted with 2 ml of 100% methanol. All samples were dried under nitrogen and stored at -80°C until LC/MS/MS analysis. Identification and quantification of 20-HETE, EETs, and DHETs were performed with a Q-trap 3200 linear ion trap quadrupole LC/MS/MS equipped with a Turbo V ion source operated in negative electrospray mode (Applied Biosystems, Foster City, CA). Multiple reaction monitoring was used with a dwell time of 25 or 50 ms for each compound, with source parameters: ion spray voltage, -4500 V; curtain gas, 40 U; ion source gas flow rate 1, 65 U and 2, 50 U; and temperature of 600ºC. Synthetic - standards (Cayman Chemical, Ann Arbor, MI) were used to obtain standard curves (5-500 pg) for for each eicosanoid and internal standard. The amounts in each sample were corrected for loss during extraction and normalized by protein amount or plasma volume.

***Measurements of vascular reactivity and endothelial function.*** Renal interlobar arteries (~230 μm, internal diameter) were cut into ring segments (2 mm) and mounted on wires in the chambers of a multivessel myograph (JP Trading, Aarhus, Denmark) filled with oxygenated Krebs' buffer (37°C). After 30 to 60 min of equilibration, the vessels were set to an internal circumference equivalent to 90% of that which they would have in vitro when relaxed under a transmural pressure of 80 mm Hg. Isometric tension was monitored continuously before and after experimental interventions. A cumulative concentration-response curve to phenylephrine ($10^{-9}$ to $5x10^{-5}$ mol/L) was constructed. The phenylephrine-induced increase in isometric tension was expressed as the percentage of the maximum contraction achieved. For measurements of the relaxing response to acetylcholine, the vascular preparations were contracted with phenylephrine to 80% of maximum contraction. When the contractile response was stabilized, a cumulative concentration-response curve to acetylcholine ($10^{-9}$ to $5x10^{-5}$ mol/L) was constructed in the presence of 20-HETE (50 nM) with and without lisinopril ( ) or losartan (). Cumulative concentration-response curve to nitroprusside ($10^{-9}$ to $5x10^{-5}$ mol/L) were performed in phenylephrine-preconstricted arteries in the presence of 20-HETE (1 μM) or 20-HEDE (1 μM). 20-HETE and 20-HEDE were added to the bath 15 min prior to phenylephrine.

***Statistical analysis.*** The data are presented as mean ± standard error (SE). Statistical significance ($p<0.05$) between the experimental groups was determined by the Fisher method of analysis for multiple comparisons. For comparison between treatment groups, the Null hypothesis was tested by a single factor analysis of variance (ANOVA; Dunnett's Multiple Comparison Test) for multiple groups or unpaired *t*-test for two groups.

RESULTS

Gene Microarray and real time PCR. **The expression of 100 genes was assessed in mRNA extracted from HMVEC treated with and without 20-HETE (1 nM) for 30 min using** the human GEarray microarray (SA Biosciences)**.** The results are summarized in Table S1. A real-time PCR analysis corroborated the microarray data; 20-HETE at a concentration of 5 nM increased ACE mRNA by 3-fold. 20-HETE-mediated induction of ACE mRNA was abolished by co-treatment with the 20-HETE antagonist 20-HEDE, demonstrating the specificity of the 20-HETE effect (Figure S1). No significant changes were observed in the expression of ACE-2, angiotensinogen, AT1R or AT2R (Figure S1).

**Plasma levels of 20-HETE, EETs and DHETs.** The total Levels of 20-HETE, EETs and DHETs were measured after alkali hydrolysis (free+esterified). There were no significant differences in the levels of these eicosanoids between GFP and CYP4A2-transduced rats. 20-HETE. As seen in Figure S1, 20-HETE levels were 1.01±0.20 and 0.78±0.22 ng/ml in lenti-VECAD-GFP and Lenti-VECAD-

CYP4A2 transduced rats, respectively. The sum of EETs+DHETs (epoxygenase activity) was 55.86±8.71 and 34.34±15.27 ng/ml in lenti-VECAD-GFP and Lenti-VECAD-CYP4A2 transduced rats, respectively. There were no significant differences in either EETs or DHETs levels.

**Effect of Lisinopril and Losartan on 20-HETE-mediated impairment of acetylcholine-induced relaxation.** As seen in Figure S2, addition of 20-HETE greatly impaired the relaxing response to acetylcholine reducing maximal relaxation from ± to ±. Addition of either lisinopril or losartan ex vivo to renal interlobar arteries from control rats attenuated 20-HETE-mediated impairment of acethylcholine-induced relaxation suggesting that the RAS system contribute to 20-HETE effect.

**Effect of 20-HETE and 20-HEDE on the relaxing response to nitroprusside in renal interlobar arteries.** As seen in Figure S3, neither 20-HETE nor 20-HEDE significantly altered the relaxing responses to nitroprusside indicating that 20-HETE does not affect NO action as previously reported [6, 7]

REFERENCES


1. Gopal VRJ, S.G.; Reddy, Y.K.; Bandyopadhyay, A.; Capdevila JHF, J.R. . A Practical, Stereospecific Route to 18-, 19-, and 20-Hydroxyeicosa-5(Z),8(Z),11(Z),14(Z)-tetraenoic Acids (18-, 19-, and 20-HETEs). *Tetrahedron Letters.* 2004;45: 2563-2565.
2. Yu M, Alonso-Galicia M, Sun CW, Roman RJ, Ono N, Hirano H, Ishimoto T, Reddy YK, Katipally KR, Reddy KM, Gopal VR, Yu J, Takhi M, Falck JR. 20-hydroxyeicosatetraenoic acid (20-HETE): structural determinants for renal vasoconstriction. *Bioorganic & medicinal chemistry.* 2003;11: 2803-2821.
3. Miyata N, Taniguchi K, Seki T, Ishimoto T, Sato-Watanabe M, Yasuda Y, Doi M, Kametani S, Tomishima Y, Ueki T, Sato M, Kameo K. HET0016, a potent and selective inhibitor of 20-HETE synthesizing enzyme. *British journal of pharmacology.* 2001;133, 325-329.
4. Edgell CJ, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc Natl Acad Sci U S A.* 1983;80: 3734-3737.
5. Inoue K, Sodhi K, Puri N, Gotlinger KH, Cao J, Rezzani R, Falck JR, Abraham NG, Laniado-Schwartzman M. Endothelial-specific CYP4A2 overexpression leads to renal injury and hypertension via increased production of 20-HETE. *Am J Physiol Renal Physiol.* 2009;297: F875-884.
6. Wang JS, Singh H, Zhang F, Ishizuka T, Deng H, Kemp R, Wolin MS, Hintze TH, Abraham NG, Nasjletti A, Laniado-Schwartzman M. Endothelial dysfunction and hypertension in rats transduced with CYP4A2 adenovirus. *Circ Res.* 2006;98: 962-969.

**7.** Singh H, Cheng J, Deng H, Kemp R, Ishizuka T, Nasjletti A, Schwartzman ML. Vascular Cytochrome P450 4A Expression and 20-Hydroxyeicosatetraenoic Acid Synthesis Contribute to Endothelial Dysfunction in Androgen-Induced Hypertension. *Hypertension.* 2007;50: 123-129.

**TABLE S1**

| Gene name | Fold change[a] | Gene name | Fold change | Gene name | Fold change |
|---|---|---|---|---|---|
| L-Selectin | 0.00 | VEGFR3 | 0.75 | Fas/Apo-1/CD95 | 0.95 |
| ETA | 0.26 | Tie-2 | 0.76 | ETRB/ETB | 0.95 |
| IL-3 | 0.29 | PLG | 0.76 | IL-8 | 0.95 |
| OCLN | 0.30 | OPG/OCIF | 0.77 | G-CSF | 0.95 |
| Integrin a5 | 0.34 | Fas ligand | 0.78 | VEGF | 0.96 |
| Bax | 0.37 | eNOS | 0.79 | BFL1 | 0.98 |
| Angiopoietin 1 | 0.43 | CGA | 0.79 | Bcl-x | 0.98 |
| NOS | 0.44 | IFN-b1 | 0.80 | Endostatin | 0.99 |
| IL-6 | 0.48 | MT1-MMP | 0.80 | Cyclophilin A | 1.00 |
| IL-11 | 0.48 | TRAIL-R3/DcR1 | 0.81 | VWF | 1.01 |
| VCAM-1 | 0.48 | IL-1b | 0.81 | RIP | 1.01 |
| TNFA | 0.52 | T-PA | 0.81 | ACE-2 | 1.01 |
| FGF2 | 0.55 | TACE/CD156b | 0.81 | Fibronectin-1 | 1.02 |
| AT1 | 0.56 | Mch2 | 0.81 | Angiopoietin-2 | 1.03 |
| IL-15 | 0.57 | SCYA5/RANTES | 0.82 | GAPDH | 1.03 |
| MCH4/FLICE2 | 0.57 | PTGIS | 0.82 | TSP1 | 1.04 |
| CD61/GP3A | 0.60 | THBD | 0.85 | PAI-1 | 1.05 |
| ET-1 | 0.63 | β-Actin | 0.85 | RPL13A | 1.06 |
| Collagenase-1 | 0.63 | Integrin b1 | 0.86 | TRAIL-R4/DcR2 | 1.06 |
| ICE | 0.64 | Cu/ZnSOD | 0.86 | MIP-3a/SCYA20 | 1.06 |
| ICAM-3 | 0.64 | TIMP1 | 0.88 | CD31 | 1.08 |
| TRAIL | 0.64 | CRADD | 0.88 | MMP-2 | 1.08 |
| Integrin aV | 0.65 | pUC18 | 0.89 | Annexin V | 1.09 |
| CASPER/FLIP | 0.65 | CPP32 | 0.89 | MCP1/SCYA2 | 1.09 |
| TF | 0.66 | PSGL1 | 0.90 | ICAM-1 | 1.09 |
| VEGFR1 | 0.67 | Cox-2 | 0.91 | ICAM-2 | 1.11 |
| uPA | 0.68 | A20 | 0.91 | PLA2G4C | 1.12 |
| ET-3 | 0.68 | Cadherin 5 | 0.91 | XDH | 1.13 |
| ENPEP | 0.68 | SCYD1 | 0.93 | Angiotensinogen | 1.22 |
| ET-2 | 0.69 | AT2 | 0.93 | GM-CSF | 1.46 |
| MMP-9 | 0.70 | FLK1/VEGFR2 | 0.93 | ELAM-1/E-selectin | 1.53 |
| ALOX5 | 0.72 | IL-14 | 0.94 | TAFI | 2.26 |
| IL-7 | 0.74 | TFPI | 0.94 | ACE | 5.76 |
| Bcl-2 | 0.74 | TFPI2 | 0.94 | | |

[a]Fold change between HMVEC cells treated and untreated with 20-HETE (1nM). Results are the mean of two separate experiments.

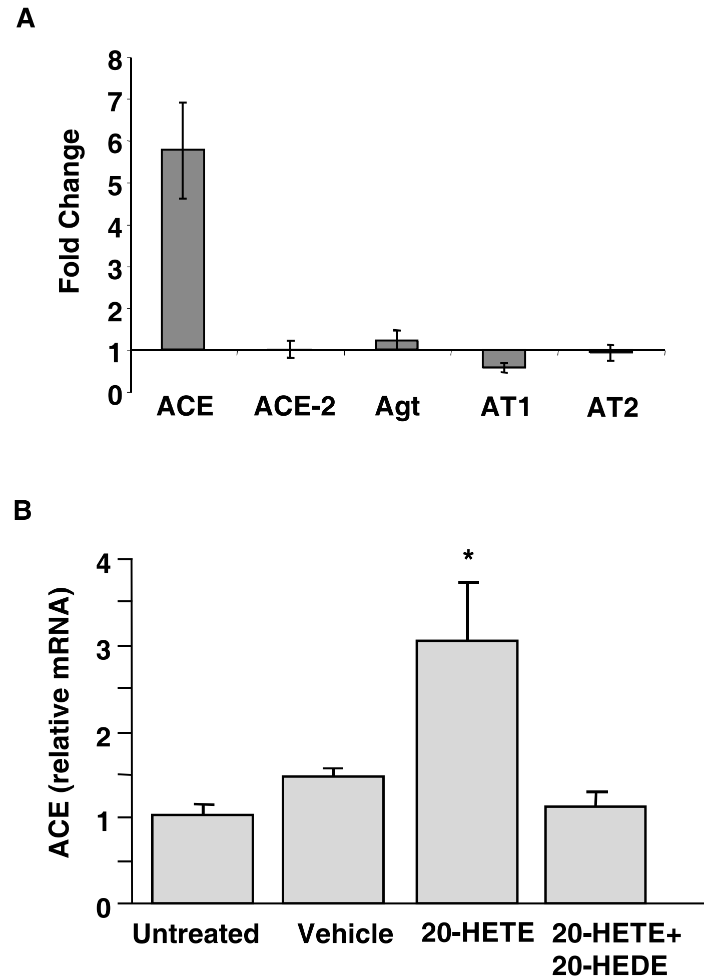


**Figure S1: (A)** Gene microarray of endothelial cells treated with 20-HETE (1 nM) for 30 min. Results are expressed as fold change from control untreated cells (n=3, *p<0.05). **(B)** Real-time PCR of ACE in endothelial cells treated with 20-HETE (5 nM) with and without 20-HEDE (5 nM) for 2 h (n=4, *p<0.05 vs control; ‡p<0.05 vs 20-HETE).

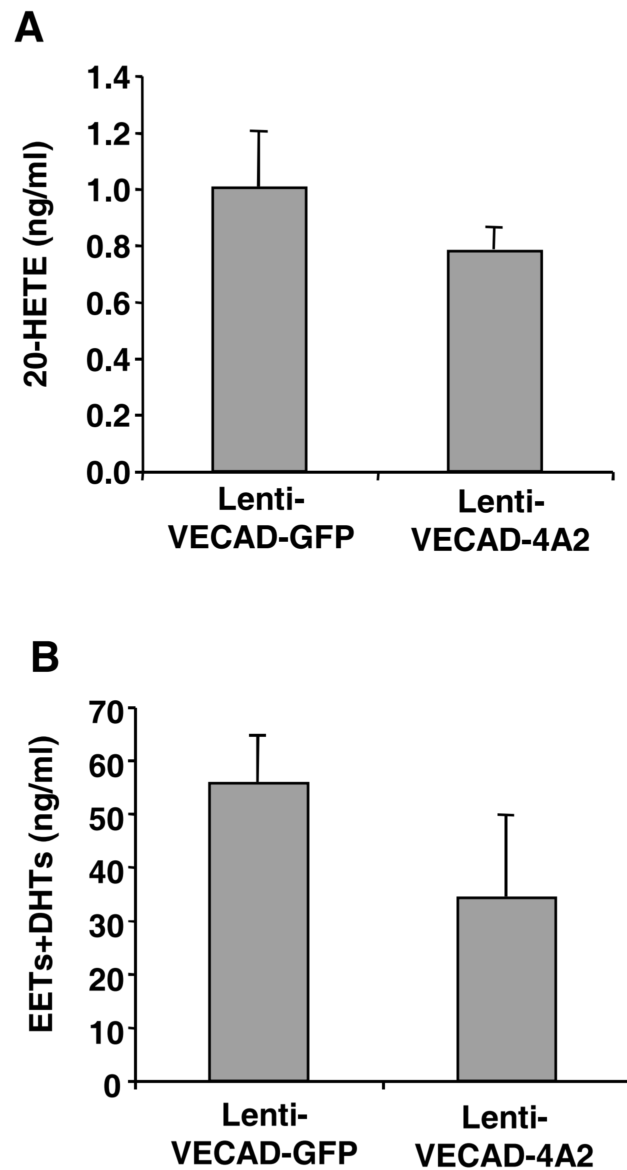


**Figure S2:** Plasma levels of 20-HETE and EETs+DHETs in lenti-VECAD-GFP and lenti-VECAD-4A2-transduced rats. Results are mean±SE, n=4-5.

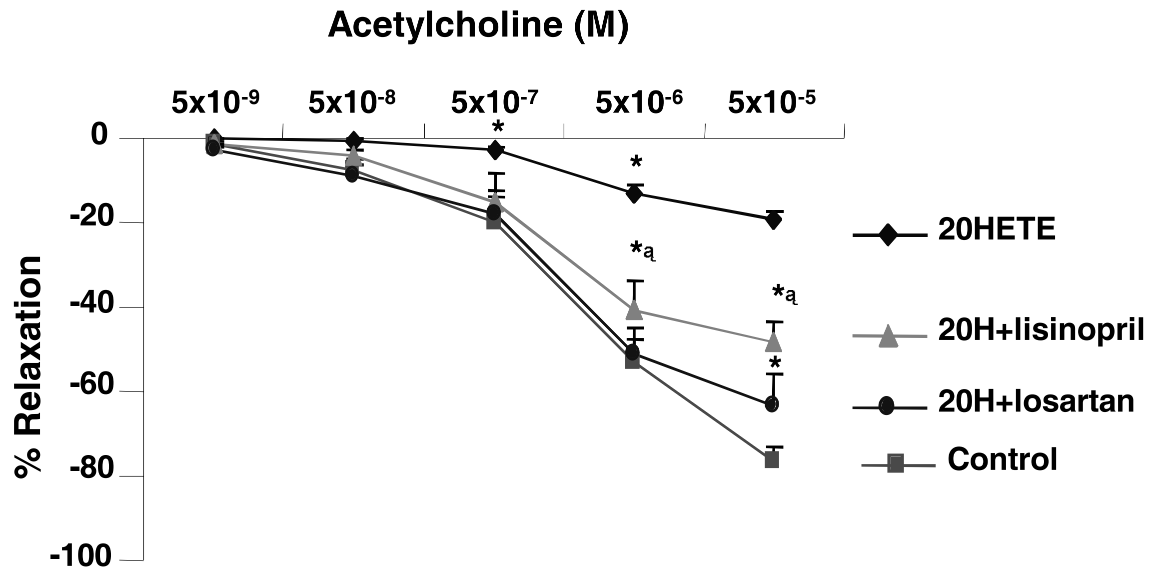


**Figure S3:** Effect of lisinopril and losartan on 20-HETE-mediated impairment of acetylcholine-induced relaxation. Interlobar arteries were isolated from SD rats (225-250 g) and mounted on wire myograph. Acetylcholine induced relaxation was assessed in the presence of 20-HETE (50 nM) with and without lisinopril ( ) or losartan ( ). Results are mean±SE; n=3; *p<0.05 from control untreated arteries; ‡p<0.05 from 20-HETE.

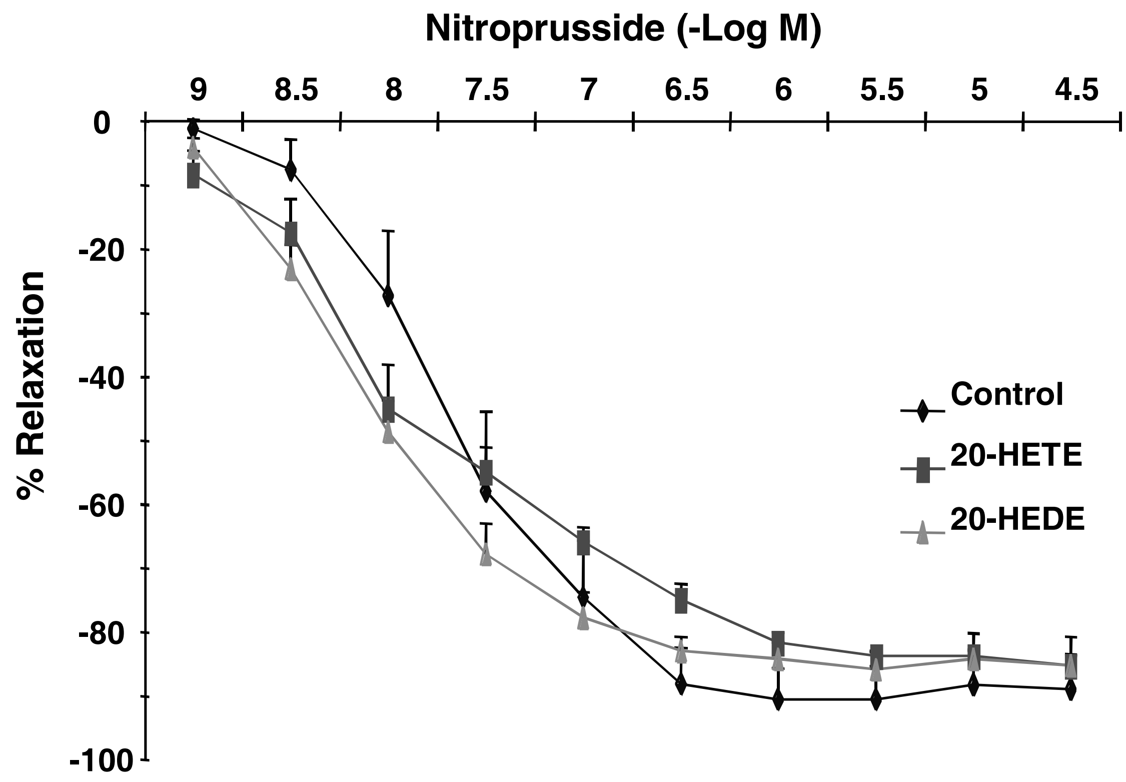
Nitroprusside (-Log M)
9
8.5
8
7.5
7
6.5
6
5.5
5
4.5
0
-20
-40
-60
-80
-100
% Relaxation
Control
20-HETE
20-HEDE

**Figure S4:** Effect of 20-HETE and 20-HEDE on the relaxing response to nitroprusside. Results are mean±SE, n=4.